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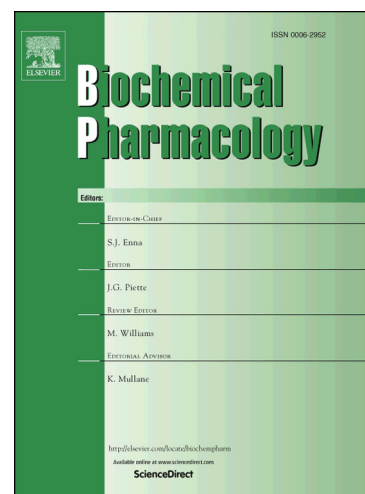
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**Basal ryanodine receptor activity suppresses autophagic flux**

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**Abstract:**

The inositol 1,4,5-trisphosphate receptors (IP<sub>3</sub>Rs) and intracellular Ca<sup>2+</sup> signaling are critically involved in regulating different steps of autophagy, a lysosomal degradation pathway. The ryanodine receptors (RyR), intracellular Ca<sup>2+</sup>-release channels mainly expressed in excitable cell types including muscle and neurons, have however not yet been extensively studied in relation to autophagy. Yet, aberrant expression and excessive activity of RyRs in these tissues has been implicated in the onset of several diseases including Alzheimer's disease, where impaired autophagy regulation contributes to the pathology. In this study, we determined whether pharmacological RyR inhibition could modulate autophagic flux in ectopic RyR-expressing models, like HEK293 cells and in cell types that endogenously express RyRs, like C2C12 myoblasts and primary hippocampal neurons. Importantly, RyR3 overexpression in HEK293 cells impaired the autophagic flux. Conversely, in all cell models tested, pharmacological inhibition of endogenous or ectopically expressed RyRs, using dantrolene or ryanodine, augmented autophagic flux by increasing lysosomal turn-over (number of autophagosomes and autolysosomes measured as mCherry-LC3 punctae/cell increased from  $70.37 \pm 7.81$  in control HEK RyR3 cells to  $111.18 \pm 7.72$  and  $98.14 \pm 7.31$  after dantrolene and ryanodine treatments, respectively). Moreover, in differentiated C2C12 cells, transmission electron microscopy demonstrated that dantrolene treatment decreased the number of early autophagic vacuoles from  $5.9 \pm 2.97$  to  $1.8 \pm 1.03$  per cellular cross section. The modulation of the autophagic flux could be linked to the functional inhibition of RyR channels as both RyR inhibitors efficiently diminished the number of cells showing spontaneous RyR3 activity in the HEK293 cell model (from  $41.14\% \pm 2.12$  in control cells to  $18.70\% \pm 2.25$  and  $9.74\% \pm 2.67$  after dantrolene and ryanodine

treatments, respectively). In conclusion, basal RyR-mediated  $\text{Ca}^{2+}$ -release events suppress autophagic flux at the level of the lysosomes.

**Keywords:**

Dantrolene, ryanodine receptor, Alzheimer's disease, autophagy, lysosome,  $\text{Ca}^{2+}$

**Chemical compounds cited in this article**

Dantrolene (PubChem CID: 6604100),

Ryanodine (PubChem CID: 11317883),

Bafilomycin A1 (PubChem CID: 6436223)

## 1. Introduction

Autophagy, a lysosomal degradation pathway, is controlled by intracellular  $\text{Ca}^{2+}$  signals arising from different organelles, including the endoplasmic reticulum (ER) and lysosomes. (1-3) At the ER, inositol 1,4,5-trisphosphate ( $\text{IP}_3$ ) receptors ( $\text{IP}_3\text{R}$ ),  $\text{IP}_3$ -gated intracellular  $\text{Ca}^{2+}$ -release channels, are well-known regulators of autophagy. (4-7)  $\text{IP}_3\text{Rs}$  have been described to be involved in both inhibitory and stimulatory effects on autophagy. For instance, it has been shown that a continuous  $\text{IP}_3\text{R}$ -mediated  $\text{Ca}^{2+}$  transfer from the ER to the mitochondrial matrix is necessary for proper energy production via mitochondrial respiration. Inhibition of  $\text{IP}_3\text{Rs}$ , and thereby preventing the ER-to-mitochondrial  $\text{Ca}^{2+}$  transfer, results in the activation of the AMP-activated protein kinase (AMPK) resulting in the induction of autophagy. (8)  $\text{IP}_3\text{Rs}$  also scaffold Beclin 1, an essential protein for phagophore formation, inhibiting autophagic flux by limiting Beclin 1 availability for Vps34-complex formation. (9) In contrast to these inhibitory actions, cytosolic  $\text{Ca}^{2+}$  elevations mediated by  $\text{IP}_3\text{Rs}$  can also drive autophagy by activation of the calmodulin-dependent protein kinase kinase- $\beta$ , an upstream activator of AMPK. (10) Moreover, starvation- and rapamycin-induced autophagic flux depends on  $\text{IP}_3\text{R}$ -mediated  $\text{Ca}^{2+}$  signaling during which  $\text{IP}_3\text{Rs}$  become sensitized by Beclin 1. (4,11) Lysosomal fusion events and other lysosomal functions, involved in autophagy, are also closely regulated by  $\text{Ca}^{2+}$ . (12) Although  $\text{Ca}^{2+}$  is necessary for vesicle fusion, lysosomal  $\text{Ca}^{2+}$  release mediated by two pore channel 2 (TPC2) was also shown to inhibit the fusion of autophagosomes and lysosomes by increasing lysosomal pH. (13,14)

In contrast to  $\text{IP}_3\text{Rs}$ , ryanodine receptors (RyR), another class of ER-located intracellular  $\text{Ca}^{2+}$ -release channels, have not been extensively studied with regards to autophagy

regulation. RyRs are more restricted in their expression pattern compared to IP<sub>3</sub>Rs, which are expressed ubiquitously. High levels of RyRs can be found in skeletal muscle (mainly RyR isoform 1), the heart (mainly RyR isoform 2) and the brain (mainly RyR isoforms 2 and 3). (15) Aberrant RyR-mediated Ca<sup>2+</sup> release has been associated with several diseases like malignant hyperthermia, (16) heart failure (17) and neurodegenerative diseases such as Huntington's disease (HD) (18) and Alzheimer's disease (AD). (19) These neurodegenerative diseases are characterized by the occurrence of protein aggregates (amyloid  $\beta$  in AD and mutant huntingtin in HD), which interfere with normal neuronal functioning. One way to clear protein aggregates is via the induction of autophagy. (20) Compounds that block voltage-gated Ca<sup>2+</sup> channels have already been shown to have beneficial effects in HD models through the stimulation of autophagy. (21) Importantly, RyR inhibition via dantrolene, a clinically-approved drug used for treating malignant hyperthermia, (22) was able to reduce the occurrence of these protein aggregates. (18,23-25) Two studies indicate a potential role for RyRs in autophagy regulation. First, RyRs were shown to contribute to autophagic cell death in neuronal hippocampal stem cells. (26) Second, it was reported that in a mouse model of neuropathic Gaucher disease RyR inhibition, using dantrolene, delayed the neurological pathology and increased survival. (27) The authors reported that dantrolene could partially reverse the observed alterations in autophagic flux, suggesting a possible role for RyRs in regulating autophagy. However, it was not further studied how RyRs influenced autophagy.

Here, we set out to identify whether modulating RyR activity can regulate autophagy, and if so, at which phase of the autophagic pathway this regulation occurs (proximal or more distal phases of the autophagic process). Using a HEK293 cell model overexpressing RyR3 (HEK RyR3), we first show that RyR3 overexpression impairs autophagic flux. Chemical RyR



inhibition using dantrolene and ryanodine reversed this phenomenon and increased the autophagic flux at the level of the lysosomes without altering the activity of upstream autophagy regulators like AMPK and mTOR or the Beclin 1-expression level. Moreover, in HEK RyR3 cells, these inhibitors efficiently suppressed spontaneous  $\text{Ca}^{2+}$ -release events. These findings were supported by experiments performed in differentiated C2C12 cells and in primary dissociated hippocampal neurons, two cell types that express endogenous RyRs. (27) Hence, these results indicate that RyR-mediated  $\text{Ca}^{2+}$ -release events suppresses autophagic flux at steps distal to the initiation of phagophore formation, and involving alteration in lysosomal function

## 2. Materials and methods

### 2.1 Antibodies and reagents

The antibodies used in this study were mouse anti-RyR clone 34C (Thermo Fisher Scientific, Ghent, Belgium), mouse anti- $\gamma$ -tubulin (Sigma-Aldrich, Overijse, Belgium), mouse anti-GAPDH (Sigma-Aldrich), mouse anti-LC3 antibody clone 5F10 (Nanotools, Teningen, Germany), mouse anti-actin (Sigma-Aldrich), mouse anti-Becclin 1 clone E8 (Santa Cruz, Dallas, USA), rabbit anti-S6 ribosomal clone 5G10, rabbit anti-phospho S6 ribosomal protein (Ser235/236), rabbit anti-AMPK $\alpha$  and rabbit anti-phospho-AMPK $\alpha$  clone 40H9 (Thr172) all from Cell Signaling Technology (Leiden, the Netherlands). Unless otherwise specified, chemicals were purchased from Sigma-Aldrich. Dantrolene was freshly prepared on the day of the experiment in water (warmed to 37°C) (1 mM stock) and filter sterilized. Dantrolene is a potent inhibitor of especially RyR1 and RyR3. In cell cultures, 10 to 50  $\mu$ M of dantrolene is a common range of concentrations used in order to assure blocking of RyRs(24,28). We therefore selected 10  $\mu$ M as working concentration. In the hippocampal neurons mainly RyR3, but also RyR2, is expressed.(29) As the RyR2 isoform is less sensitive to dantrolene(30) a higher concentration (20  $\mu$ M) was also included in these experiments in order to validate the effects of dantrolene. Bafilomycin A1 (Santa Cruz) and ryanodine (Enzo Life Sciences, Brussels, Belgium) were dissolved in DMSO. Bafilomycin A1 (100 nM) is commonly used to study autophagy, as at this concentration it is well known to potently block the V-type ATP-ases in the lysosomes, and thus to inhibit autophagic flux.(31) Ryanodine has a bimodal effect on the RyR, where low concentrations (nanomolar range) lock the channel in an open sub-conductance state, while high concentrations (micromolar range)

inhibit RyR-mediated  $\text{Ca}^{2+}$  release.(32,33) The ryanodine concentration (20  $\mu\text{M}$ ) used in this study has been used in many studies to fully inhibit RyR activity.

## 2.2 Cell culture and treatments lysate preparation

Unless otherwise specified all cell culture media and supplements were obtained from Thermo Fisher Scientific. Cell lines were at least monthly checked for mycoplasma infections. Cell lines were passaged no more than 20 times before thawing fresh batches. All experiments were performed in mycoplasma-free cells. C2C12 cells were obtained from the ATCC (Molsheim Cedex, France) and were maintained and grown in growth medium (Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal calf serum, 1% non-essential amino acids (NEAA), 100 IU/ml penicillin, 100  $\mu\text{g}/\text{ml}$  streptomycin, 2 mM Glutamax and 0.5% chicken embryo extract (Biotrend, Köln, Germany). Differentiation of C2C12 cells was started by changing to differentiation medium (DMEM with 1% NEAA, 100 IU/ml penicillin, 100  $\mu\text{g}/\text{ml}$  streptomycin, 2 mM Glutamax, 1% horse serum and 0.5% insulin-transferrin-sodium selenite) when cells reached approximately 80% confluence. HEK293 cells were obtained from DSMZ (Braunschweig, Germany). The HEK RyR3 cells were a kind gift of Dr. Vincenzo Sorrentino. (34) HEK293 cells stably expressing either the empty pcDNA3.1(-) vector (HEK control), or this vector containing RyR3 (HEK RyR3) were cultured as described previously. (35) All cells were treated in the same manner. One day before the experiment the medium was changed. On the day of the experiment, the medium was also replaced. Two hours later, cells were treated with vehicle or the indicated concentrations of dantrolene or ryanodine in fresh medium. When dantrolene and ryanodine were used in the same experiment, DMSO was also added to the dantrolene treatment in

order to be able to compare the treatments. Treatments lasted for three hours, and when indicated bafilomycin A1 (100 nM) was added for the final hour before harvesting the cells for analysis. After the treatments, cells were washed with phosphate-buffered saline (PBS) (Life Technologies), scraped and centrifuged at 500 x g. Pellets were solubilized in a CHAPS-based lysis buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 2 mM EDTA, 50 mM NaF (S7920), 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1% CHAPS and protease inhibitor tablets (Roche, Basel, Switzerland) for 30 min at 4°C. After centrifuging at 4000 x g the supernatant was collected and used for immunoblot analysis. (35)

### **2.3 Culture and treatment of dissociated hippocampal neurons**

Dissociated rat hippocampal neurons were obtained according to ethically approved guidelines and maintained as previously reported. (35) After fifteen days of culturing the dissociated hippocampal neurons, half of the medium was exchanged for fresh medium (neurobasal medium containing 2 mM Glutamax, 100 IU/ml penicillin, 100 µg/ml streptomycin and 2% B27 supplement, all from Life Technologies). Two hours later, half of the medium was taken from the neuronal cultures and was used to prepare experimental conditions as indicated in the text. Re-addition of this medium resulted in the final concentrations of vehicle, dantrolene and ryanodine. Three hours later, cells were harvested. When indicated bafilomycin A1 (100 nM) was added the last hour before harvesting the cells.

## 2.4 mCherry-LC3 puncta and quantification

HEK RyR3 cells were seeded in two-chamber slides and transfected two days later with pcDNA3.1(-) vector encoding mCherry-LC3 utilizing the X-tremeGENE™ HP DNA transfection reagent (Sigma-Aldrich) according to the manufacturer's instructions. A day later, the medium was changed and the next day treatments were performed as described above. Subsequently, cells were washed twice with PBS and fixed for 10 min with 4% paraformaldehyde in PBS at 4°C. Cells were extensively washed with PBS and stored in PBS at 4°C until imaged. Confocal images were taken utilizing a 63x objective on a Zeiss LSM510 microscope (Jena, Germany). The amount of mCherry-LC3 puncta in each cell were calculated utilizing the WatershedCounting3D plug-in for ImageJ. (36)

## 2.5 Outer ER membrane-targeted $\text{Ca}^{2+}$ measurements

The genetically encoded outer ER membrane-targeted (OER)  $\text{Ca}^{2+}$  indicator OER-GCaMP6f was expressed in HEK RyR3 cells under the control of the cytomegalovirus promoter (37).

HEK RyR3 cells were seeded in round four-chamber slides, and after 24 hours, transfected with the OER-GCaMP6f probe using the X-tremeGENE™ HP DNA transfection reagent according to the manufacturer's instructions. The medium was changed the next day, and the following morning cells were treated as described in section 2.2. After the 3 hours treatment, the medium was replaced with a modified Krebs solution (135 mM NaCl, 6.2 mM KCl, 1.2 mM  $\text{MgCl}_2$ , 12 mM HEPES, pH 7.3, 11.5 mM glucose and 2 mM  $\text{CaCl}_2$ ) supplemented with either DMSO, 10  $\mu\text{M}$  dantrolene or 20  $\mu\text{M}$  ryanodine before starting the  $\text{Ca}^{2+}$  measurements.

Single-cell  $\text{Ca}^{2+}$  measurements were performed in OER-GCaMP6f-positive cells using a Zeiss Axio Observer Z1 Inverted Microscope equipped with a 20x air objective and a high-speed digital camera (Axiocam Hsm, Zeiss, Jena, Germany). Images were taken at 5 hertz. Spontaneous peri-ER  $\text{Ca}^{2+}$ -release events were measured for 300 sec. Using an automated perfusion system, the RyR agonist caffeine was subsequently added at a concentration of 1.5 mM (for 120 sec) and 10 mM (for 70 sec).

## **2.6 Transmission electron microscopy**

C2C12 cells were cultured, differentiated for 5 days and treated as stated in section 2.2. After the 3 hours treatments, the cells were trypsinized (Thermo Fisher Scientific), collected and pelleted at 500 x g. The pellets were washed once with PBS prior to fixation in 0.1 M sodium cacodylate-buffered (pH 7.4) 2.5% glutaraldehyde solution. Further handling of the samples and transmission electron microscopy (TEM) was performed as described. (38)

## **2.7 Statistical analysis**

Sample size and normalization of data are indicated in the figure legends. When three groups were compared an ANOVA with Dunnett's multiple comparison post hoc test was performed. When two groups were compared a two-tailed unpaired or paired student's t-test was used. Differences between groups were considered significant (\*) when  $P < 0.05$  (P values

for the ANOVA and student's t-test are given in the figures). \*\* and \*\*\* indicate P values <0.01 and <0.001, respectively. The exact P values are provided, unless  $P < 0.0001$ .

### 3. Results

#### 3.1 Inhibition of ectopically expressed RyR3 in HEK293 cells stimulates autophagic flux

In order to determine the potential effects of the RyR on autophagy and to decipher the autophagic pathway involved, we used HEK293 cells stably expressing the RyR3 channel (HEK RyR3). RyR protein levels were detected via immunoblot using a RyR antibody that recognizes all RyR isoforms (Figure 1A). A strong RyR expression was observed in HEK RyR3 cells whereas no RyR expression was detected in HEK293 cells expressing the empty vector (HEK control). Autophagy was monitored using the marker LC3-II, the lipidated form of LC3-I, which is incorporated into the autophagosomes. Overexpression of RyR3 resulted in increased levels of LC3-II compared to the HEK control cells (Figure 1A). To further document the contribution of RyRs in regulating autophagy, HEK RyR3 cells were treated for three hours with two RyR inhibitors: dantrolene (10  $\mu$ M) or a high dose of ryanodine (20  $\mu$ M). Both dantrolene and ryanodine treatments reduced LC3-II levels compared to the vehicle control (Figure 1B) in HEK RyR3 cells. This indicates that the increase in LC3-II upon is due to RyR3 overexpression as this can be counteracted by RyR inhibitors. Two possibilities exist for the decline in LC3-II levels upon RyR inhibition.<sup>(31)</sup> RyR inhibition might lead to a decrease in autophagic flux, particularly in the steps preceding LC3-II and autophagosome formation. Alternatively, RyR inhibition might lead to an increased clearance of LC3-II and autophagosomes due to fusion with the lysosomes, corresponding to an increased autophagic flux. To further document this we used the RyR inhibitors in the presence of the lysosomal inhibitor bafilomycin A1, which was added during the last hour of the treatments. Neither dantrolene nor ryanodine diminished the amount of LC3-II that



accumulated in cells incubated with bafilomycin A1. Bafilomycin A1 halts autophagic flux at the step of autophagosomal and lysosomal fusion. These data therefore suggest that dantrolene and ryanodine decrease LC3-II, not by preventing its formation, but by increasing its degradation at a step distal to autophagosomal and lysosomal fusion (Figure 1B). This indicates that the observed decrease in LC3-II protein levels upon RyR inhibition is due to an increased clearance of LC3-II by lysosomal degradation, and thus an increased autophagic flux.

Hence, these results indicate that RyR3 inhibition increases LC3-II breakdown in the autolysosomes. An increase in the number of autolysosomes upon RyR inhibition could account for these observations. We therefore assessed the number of mCherry-LC3 puncta in HEK RyR3 cells exposed to the vehicle, dantrolene, or ryanodine. We opted to use mCherry-LC3 puncta, because mCherry, in contrast to green fluorescent protein (GFP), is not quenched by acidification of the lysosomes. As a consequence, mCherry-LC3 allows quantification of the total number of autophagosomes and autolysosomes without using bafilomycin A1, which also affects  $\text{Ca}^{2+}$  homeostasis at the lysosomes.(39) Moreover, HEK RyR3 cells displayed a prominent autofluorescence upon excitation at 480 nm, thereby hampering the use of GFP-LC3 for quantitative puncta determination. Figure 1C (left panel) shows an example of HEK RyR3 cells transiently transfected with mCherry-LC3 and treated for three hours with the vehicle or the indicated amounts of dantrolene or ryanodine. Quantification of the total number of mCherry-puncta per cell for each condition (Figure 1C, right panel) revealed that RyR3 inhibition increased the number of mCherry-LC3 puncta and thus the number of autophagosomes and autolysosomes.

### 3.2 RyR3 inhibition does not alter the proximal/intermediate steps of autophagy

Using mCherry-LC3, it is not possible to determine whether the observed increase in mCherry-LC3 punctae is due to an increase in autophagosomes, autolysosomes or a combination of the two. In order to elucidate these aspects in more detail, we examined different autophagy markers in HEK RyR3 cells treated with dantrolene or ryanodine (Figure 2). We studied markers involved in the initiation of the autophagic pathway (e.g. AMPK and mechanistic target of rapamycin (mTOR) activity).<sup>(31)</sup> AMPK activity (assessed via its phosphorylation state) and mTOR activity (assessed via the phosphorylation of S6 ribosomal protein (S6RP) a downstream target of mTOR) were not altered upon treatment of cells with dantrolene or ryanodine. This indicated that RyR inhibition in these cells did not impair mitochondrial bio-energetics or nutrient-sensing mechanisms. Also, total Beclin 1 levels, a protein involved in the early events of autophagosome formation, were not affected by dantrolene or ryanodine. Combining this information with the data from section 3.1 indicates that RyR inhibition appears to enhance the turn-over of autophagosomal cargo without affecting the earlier steps of autophagy involved in the production and formation of autophagosomes.

### 3.3 Dantrolene and ryanodine inhibit spontaneous RyR3-mediated $\text{Ca}^{2+}$ release in the conditions used for analyzing autophagic flux

As the impact of the RyR inhibitors on basal autophagic flux was determined in the absence of RyR agonist, we aimed to determine whether these inhibitors were able to suppress

spontaneous RyR-mediated  $\text{Ca}^{2+}$  release in exactly the same conditions used for determining their impact on autophagic flux. We therefore used a recently developed genetically encoded  $\text{Ca}^{2+}$  indicator (GECI), based on GCaMP6f, which is targeted to the outer side of the ER membrane (OER-GCaMP6f). (37) This GECI is able to detect small  $\text{Ca}^{2+}$ -release events at subcellular resolution in the close proximity of the ER membrane. We previously showed that spontaneous RyR3-mediated  $\text{Ca}^{2+}$ -release events occur in HEK RyR3 cells. (40) Here, we set out to assess whether dantrolene and ryanodine could inhibit these spontaneous events under conditions used for measuring their impact on autophagy. Spontaneous peri-ER  $\text{Ca}^{2+}$ -release events were measured in OER-GCaMP6f-transfected HEK RyR3 cells (Figure 3). Images were taken at 5 hertz, enabling a sufficiently high time resolution to also register small RyR-mediated- $\text{Ca}^{2+}$  release events. In order to be classified as a *bona fide* spontaneous  $\text{Ca}^{2+}$ -release event, we established that the maximum peak amplitude of the  $\text{Ca}^{2+}$  release should be at least 0.02 units above baseline and/or should form a  $\text{Ca}^{2+}$  transient lasting longer than 5 secs. Figure 3A shows a typical experiment. OER-GCaMP6f-transfected HEK RyR3 cells were treated for three hours with DMSO, dantrolene (10  $\mu\text{M}$ ) or ryanodine (20  $\mu\text{M}$ ) before measuring spontaneous  $\text{Ca}^{2+}$  release. With regard to the DMSO-treated HEK RyR3 cells,  $41.14\% \pm 2.12$  showed spontaneous activity within a time frame of 5 minutes. A three hour treatment with dantrolene or ryanodine, strongly reduced the proportion of spontaneously active cells to  $18.70\% \pm 2.25$  and  $9.74\% \pm 2.67$  respectively (Figure 3A and B ). In order to show the presence of functional RyR3, the cells were subsequently treated with caffeine. DMSO- and dantrolene-treated cells responded to 1.5 mM caffeine with a pronounced RyR-mediated  $\text{Ca}^{2+}$  release. In the ryanodine-treated cells, however, even a 10 mM dose of caffeine only triggered a small RyR-mediated  $\text{Ca}^{2+}$  release. Hence, dantrolene and ryanodine efficiently inhibited spontaneous RyR-mediated  $\text{Ca}^{2+}$  release in the HEK RyR3 cells under conditions in which both inhibitors were able to augment autophagic flux.

### 3.4 Dantrolene stimulates autophagic flux in differentiated C2C12 cells

The HEK RyR3 cells are a robust and tractable cellular model to study the effects of RyR overexpression on autophagy. However, overexpressing RyRs in cells that do not endogenously express this channel may lead to severe alterations in cellular processes without major physiological relevance. Therefore, we performed experiments in differentiated C2C12 cells and primary dissociated hippocampal neurons, both expressing RyRs endogenously. Since dantrolene and ryanodine treatments produced similar results, and dantrolene is in clinical use, we decided to continue these experiments with dantrolene. Undifferentiated/proliferating C2C12 myoblasts do not display endogenous RyR expression. However, upon differentiation into myotubes, these cells express large amounts of RyR1, allowing to determine the effects of inhibiting endogenous RyR channels on autophagy in a physiologically relevant cell model. Similar experiments as in the HEK RyR3 cells were performed. Undifferentiated and 5-days differentiated C2C12 cells were treated with dantrolene for three hours, after which autophagic markers were assessed (Figure 4 A). After 5 days of differentiation, RyR expression could be detected (Figure 4A, top). Similarly as in HEK RyR3 cells, dantrolene treatment resulted in a decrease in LC3-II levels in differentiated C2C12 cells, but not in undifferentiated/proliferating C2C12 cells. This not only confirms our previous findings obtained in HEK RyR cells but also indicates that the effects of dantrolene on autophagy are mediated through RyR inhibition. Similarly to our results obtained with HEK RyR cells (Figure 1), dantrolene was not able to provoke a decrease in LC3-II levels if the C2C12 cells were exposed to bafilomycin A1. Moreover, dantrolene treatment of the 5-days differentiated C2C12 cells did not affect the activity/level of several proximal autophagic markers like phosphorylated AMPK, mTOR-substrate S6RP phosphorylation and total Beclin 1 levels (Figure 4B). Because of difficulties in combining cellular differentiation

with efficient overexpression of constructs to monitor autophagy in C2C12 cells, we performed TEM as an additional technique (Figure 4C). C2C12 cells were differentiated for 5 days and then treated similarly as above with dantrolene for three hours before preparing the samples for TEM. The number of early autophagic vacuoles, vacuolar structures clearly containing cellular components delineated by a double membrane (white arrows in Figure 4C), were determined. This TEM analysis revealed that the DMSO-treated cells contained significantly more early autophagic vacuoles per cellular cross-section compared to the dantrolene-treated cells ( $5.9 \pm 2.97$  to  $1.8 \pm 1.03$  early autophagic vacuoles per cellular cross-section respectively). These experiments suggest that the presence of functional RyR channels results in the accumulation of early autophagic vacuoles, which can be cleared by RyR inhibition. Thus, these results are fully compatible with an increase in autophagic flux due to a stimulation of autophagosome clearance via the lysosomes upon inhibition of basal RyR activity.

### **3.5 RyR inhibition stimulates autophagic flux in dissociated hippocampal neurons**

Finally, we aimed to determine the effect of dantrolene on autophagy in primary dissociated hippocampal neurons, given the previous use of dantrolene in models for neurodegeneration. Therefore, rat hippocampi were harvested at embryonic day 18 from which neurons were cultured. Fifteen days later, the resulting neurons were treated with dantrolene for three hours and autophagy was monitored using the LC3-II marker (Figure 5). Treating hippocampal neurons with dantrolene (10 or 20  $\mu$ M) showed a decrease in the amount of LC3-II protein which was abolished when bafilomycin A1 was added during the last hour of the treatments. This suggests that also in the hippocampal neurons, RyR inhibition leads to an increased

clearance of LC3-II from the autophagosomes by lysosomal degradation, confirming that dantrolene treatment accelerates the autophagic flux.

#### 4. Discussion

The major finding of this study is that basal, spontaneous RyR activity suppresses autophagic flux by reducing autophagosomal-lysosomal turnover. Application of RyR inhibitors to cell models that ectopically or endogenously express RyRs results in a decrease in LC3-II that could be attributed to an increase in autophagosomal-lysosomal turnover. Thus, chemical inhibition of RyRs can boost autophagic flux and clearance of autophagosomal cargo in the lysosomes in a variety of RyR-expressing cell systems.

Currently, the regulation of autophagosomal-lysosomal fusion events by intracellular  $\text{Ca}^{2+}$  remains poorly understood. An interesting hypothesis for this regulation is based on intimate connections existing between lysosomes and the ER in general, but also with the RyRs in particular. (3,41-43) RyRs that are in close proximity to lysosomes can be activated by  $\text{Ca}^{2+}$  - induced  $\text{Ca}^{2+}$  release, triggered by small  $\text{Ca}^{2+}$  releases from the lysosomes thereby leading to an amplification of the signal. (44) Although  $\text{Ca}^{2+}$  is involved in lysosomal fusion events, excessive  $\text{Ca}^{2+}$  levels could hamper this process. For instance, it was reported that after TPC2 overexpression, TPC2-mediated lysosomal  $\text{Ca}^{2+}$  release impaired autophagosomal/lysosomal turnover. (13,14) These lysosomal  $\text{Ca}^{2+}$  releases could trigger RyR activation thereby amplifying the  $\text{Ca}^{2+}$  signal which in turn could hamper the fusion of autophagosomes with lysosomes and prevent the degradation of autophagosomal cargo. The single-cell  $\text{Ca}^{2+}$  measurements performed in section 3.3 showed that inhibiting RyR3 activity reduced spontaneous ER  $\text{Ca}^{2+}$  release. Combining these  $\text{Ca}^{2+}$  measurements with the observed increased autophagic flux in these and the other tested cells (sections 3.1, 3.4 and 3.5)

induced by RyR inhibition suggests a role for basal RyR-mediated  $\text{Ca}^{2+}$  release in the regulation of autophagosomal clearance via the lysosomes.

Combining our findings on RyRs with the previous work from the Foscett lab on  $\text{IP}_3\text{Rs}$  (8), it seems that the spontaneous activity of both types of intracellular  $\text{Ca}^{2+}$ -release channels can suppress autophagic flux. While  $\text{IP}_3\text{Rs}$  act at the mitochondrial level by increasing mitochondrial bio-energetics and thus ATP output, resulting in a decline of AMPK activity, a positive regulator of autophagy (8), RyRs likely act more distal in the autophagy process at the level of the lysosomes by suppressing the clearance of autophagosomes. At this time we cannot fully exclude that RyR activity may also affect earlier steps in the autophagy process via influencing other parameters/targets beyond the scope of this study. In any case, our new findings further support the relevance of  $\text{Ca}^{2+}$  microdomains in the regulation of cellular processes like autophagy. (3)

Our findings might also be of importance to understand the contribution of dysregulated RyR expression and function for the pathogenesis of AD. During AD, lysosomal functioning is hampered, resulting in a reduced clearance of autophagosomal cargo at the lysosomes.(45,46)

At the same time, increased levels of RyR3 as well as excessive RyR-mediated  $\text{Ca}^{2+}$  release have been shown to be early/intermediate events in AD pathogenesis in several models.

(19,23-25,47) Blocking RyRs utilizing dantrolene has been reported to have beneficial effects in these mouse models by reducing pathological protein aggregates and memory impairment.

(23-25) However, it should be noted that in contrast to the beneficial effects in AD mouse models, prolonged feeding of dantrolene to AD mice was also linked to an increased amyloid load. (48) Differences in the amounts, administration, mouse models and duration of the



dantrolene treatments have been mentioned as potential underlying explanations for these discrepancies.(47,49) The findings described in this paper would fit with a beneficial effect of RyR inhibition on AD progression. A decreased autophagic flux was seen upon overexpression of RyR3 in HEK293 cells. This could be reversed by inhibiting the RyR with dantrolene (section 3.1). These findings were confirmed in primary dissociated hippocampal neurons indicating that a similar regulation of autophagic flux occurs in neurons (section 3.4). Stimulation of autophagic flux via RyR inhibition may thus in part account for the beneficial effects described for dantrolene in AD.(23-25) However, more research will be necessary to investigate this further. In addition, it is currently unknown how prolonged RyR inhibition would impact the autophagic process. It will therefore be interesting and necessary to study the effects of long- term dantrolene feeding on autophagy in neurons.

Finally, our results further highlight the importance of performing autophagic flux experiments using lysosomal inhibitors and performing independent approaches to adequately interpret altered LC3-II levels in terms of autophagy. Indeed, cells overexpressing RyR3 display increased LC3-II levels compared to control counterparts, which actually is due to an inhibition of autophagic flux at a distal step instead of an induction of autophagy.

In conclusion, we show that overexpression of RyR3 elevates LC3-II levels. This could be attributed to a decreased clearance of autophagosomes as acute inhibition of RyR3 increased the autophagic flux by promoting the turn-over of autophagosomal cargo at the level of the lysosomes. These findings were confirmed in differentiated C2C12 cells and dissociated hippocampal neurons, both expressing endogenous RyRs. Hence, this indicates that spontaneous  $\text{Ca}^{2+}$ -release events mediated by RyR channels may negatively regulate the autophagy process at the lysosomes.

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**The authors declare no competing interests.**

## 5. References

1. Decuypere JP, Bultynck G, Parys JB. A dual role for Ca(2+) in autophagy regulation. *Cell Calcium* 2011; (50): 242-50.
2. Sun F, Xu X, Wang X, Zhang B. Regulation of autophagy by Ca<sup>2+</sup>. *Tumour Biol* 2016; 10.1007/s13277-016-5353-y
3. La Rovere RM, Roest G, Bultynck G, Parys JB. Intracellular Ca(2+) signaling and Ca(2+) microdomains in the control of cell survival, apoptosis and autophagy. *Cell Calcium* 2016; (60): 74-87.
4. Decuypere JP, Welkenhuyzen K, Luyten T, Ponsaerts R, Dewaele M, Molgo J, et al. Ins(1,4,5)P3 receptor-mediated Ca<sup>2+</sup> signaling and autophagy induction are interrelated. *Autophagy* 2011; (7): 1472-89.
5. Messai Y, Noman MZ, Hasmim M, Janji B, Tittarelli A, Boutet M, et al. ITPR1 protects renal cancer cells against natural killer cells by inducing autophagy. *Cancer Res* 2014; (74): 6820-32.
6. Sarkar S, Floto RA, Berger Z, Imarisio S, Cordenier A, Pasco M, et al. Lithium induces autophagy by inhibiting inositol monophosphatase. *J Cell Biol* 2005; (170): 1101-11.
7. Hamada K, Terauchi A, Nakamura K, Higo T, Nukina N, Matsumoto N, et al. Aberrant calcium signaling by transglutaminase-mediated posttranslational modification of inositol 1,4,5-trisphosphate receptors. *Proc Natl Acad Sci U S A* 2014; (111): E3966-75.
8. Cardenas C, Miller RA, Smith I, Bui T, Molgo J, Muller M, et al. Essential regulation of cell bioenergetics by constitutive InsP3 receptor Ca<sup>2+</sup> transfer to mitochondria. *Cell* 2010; (142): 270-83.
9. Criollo A, Maiuri MC, Tasdemir E, Vitale I, Fiebig AA, Andrews D, et al. Regulation of autophagy by the inositol trisphosphate receptor. *Cell Death Differ* 2007; (14): 1029-39.

10. Hoyer-Hansen M, Bastholm L, Szyniarowski P, Campanella M, Szabadkai G, Farkas T, et al. Control of macroautophagy by calcium, calmodulin-dependent kinase kinase-beta, and Bcl-2. *Mol Cell* 2007; (25): 193-205.
11. Decuypere JP, Kindt D, Luyten T, Welkenhuyzen K, Missiaen L, De Smedt H, et al. mTOR-Controlled Autophagy Requires Intracellular Ca(2+) Signaling. *PLoS One* 2013; (8): e61020.
12. Morgan AJ, Platt FM, Lloyd-Evans E, Galione A. Molecular mechanisms of endolysosomal Ca<sup>2+</sup> signalling in health and disease. *Biochem J* 2011; (439): 349-74.
13. Lu Y, Hao B, Graeff R, Yue J. NAADP/TPC2/Ca(2+) Signaling Inhibits Autophagy. *Commun Integr Biol* 2013; (6): e27595.
14. Lu Y, Hao BX, Graeff R, Wong CW, Wu WT, Yue J. Two pore channel 2 (TPC2) inhibits autophagosomal-lysosomal fusion by alkalinizing lysosomal pH. *J Biol Chem* 2013; (288): 24247-63.
15. Lanner JT, Georgiou DK, Joshi AD, Hamilton SL. Ryanodine receptors: structure, expression, molecular details, and function in calcium release. *Cold Spring Harb Perspect Biol* 2010; (2): a003996.
16. Stephens J, Schiemann AH, Roesl C, Miller D, Massey S, Pollock N, et al. Functional analysis of RYR1 variants linked to malignant hyperthermia. *Temperature (Austin)* 2016; (3): 328-39.
17. Li N, Wang Q, Sibrian-Vazquez M, Klipp RC, Reynolds JO, Word TA, et al. Treatment of catecholaminergic polymorphic ventricular tachycardia in mice using novel RyR2-modifying drugs. *Int J Cardiol* 2016; 10.1016/j.ijcard.2016.10.078
18. Chen X, Wu J, Lvovskaya S, Herndon E, Supnet C, Bezprozvanny I. Dantrolene is neuroprotective in Huntington's disease transgenic mouse model. *Mol Neurodegener* 2011; (6): 81.
19. Bruno AM, Huang JY, Bennett DA, Marr RA, Hastings ML, Stutzmann GE. Altered ryanodine receptor expression in mild cognitive impairment and Alzheimer's disease. *Neurobiol Aging* 2012; (33): 1001 e1-6.
20. Cho MH, Cho K, Kang HJ, Jeon EY, Kim HS, Kwon HJ, et al. Autophagy in microglia degrades extracellular beta-amyloid fibrils and regulates the NLRP3 inflammasome. *Autophagy* 2014; (10): 1761-75.

21. Williams A, Sarkar S, Cuddon P, Ttofi EK, Saiki S, Siddiqi FH, et al. Novel targets for Huntington's disease in an mTOR-independent autophagy pathway. *Nat Chem Biol* 2008; (4): 295-305.
22. Butala BN, Kang A, Guron J, Brandom BW. Long term oral Dantrolene Improved Muscular Symptoms in a Malignant Hyperthermia Susceptible Individual. *J Neuromuscul Dis* 2016; (3): 115-9.
23. Chakroborty S, Briggs C, Miller MB, Goussakov I, Schneider C, Kim J, et al. Stabilizing ER Ca<sup>2+</sup> channel function as an early preventative strategy for Alzheimer's disease. *PLoS One* 2012; (7): e52056.
24. Oules B, Del Prete D, Greco B, Zhang X, Lauritzen I, Sevalle J, et al. Ryanodine receptor blockade reduces amyloid-beta load and memory impairments in Tg2576 mouse model of Alzheimer disease. *J Neurosci* 2012; (32): 11820-34.
25. Peng J, Liang G, Inan S, Wu Z, Joseph DJ, Meng Q, et al. Dantrolene ameliorates cognitive decline and neuropathology in Alzheimer triple transgenic mice. *Neurosci Lett* 2012; (516): 274-9.
26. Chung KM, Jeong EJ, Park H, An HK, Yu SW. Mediation of Autophagic Cell Death by Type 3 Ryanodine Receptor (RyR3) in Adult Hippocampal Neural Stem Cells. *Front Cell Neurosci* 2016; (10): 116.
27. Liou B, Peng Y, Li R, Inskeep V, Zhang W, Quinn B, et al. Modulating ryanodine receptors with dantrolene attenuates neuronopathic phenotype in Gaucher disease mice. *Hum Mol Genet* 2016; 10.1093/hmg/ddw322
28. Zhao F, Li P, Chen SR, Louis CF, Fruen BR. Dantrolene inhibition of ryanodine receptor Ca<sup>2+</sup> release channels. Molecular mechanism and isoform selectivity. *J Biol Chem* 2001; (276): 13810-6.
29. Martin C, Chapman KE, Seckl JR, Ashley RH. Partial cloning and differential expression of ryanodine receptor/calcium-release channel genes in human tissues including the hippocampus and cerebellum. *Neuroscience* 1998; (85): 205-16.
30. Tian Q, Katz AM, Kim DH. Effects of azumolene on doxorubicin-induced Ca<sup>2+</sup> release from skeletal and cardiac muscle sarcoplasmic reticulum. *Biochim Biophys Acta* 1991; (1094): 27-34.

31. Klionsky DJ, Abdelmohsen K, Abe A, Abedin MJ, Abeliovich H, Acevedo Arozena A, et al. Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition). *Autophagy* 2016; (12): 1-222.
32. Hasselbach W, Migala A. Activation and inhibition of the calcium gate of sarcoplasmic reticulum by high-affinity ryanodine binding. *FEBS Lett* 1987; (221): 119-23.
33. Coronado R, Morrisette J, Sukhareva M, Vaughan DM. Structure and function of ryanodine receptors. *Am J Physiol* 1994; (266): C1485-504.
34. Rossi D, Simeoni I, Micheli M, Bootman M, Lipp P, Allen PD, et al. RyR1 and RyR3 isoforms provide distinct intracellular  $\text{Ca}^{2+}$  signals in HEK 293 cells. *J Cell Sci* 2002; (115): 2497-504.
35. Vervliet T, Decrock E, Molgo J, Sorrentino V, Missiaen L, Leybaert L, et al. Bcl-2 binds to and inhibits ryanodine receptors. *J Cell Sci* 2014; (127): 2782-92.
36. Gniadek TJ, Warren G. WatershedCounting3D: a new method for segmenting and counting punctate structures from confocal image data. *Traffic* 2007; (8): 339-46.
37. Niwa F, Sakuragi S, Kobayashi A, Takagi S, Oda Y, Bannai H, et al. Dissection of local  $\text{Ca}^{2+}$  signals inside cytosol by ER-targeted  $\text{Ca}^{2+}$  indicator. *Biochem Biophys Res Commun* 2016; (479): 67-73.
38. Martinet W, Timmermans JP, De Meyer GR. Methods to assess autophagy in situ--transmission electron microscopy versus immunohistochemistry. *Methods Enzymol* 2014; (543): 89-114.
39. McCue HV, Wardyn JD, Burgoyne RD, Haynes LP. Generation and characterization of a lysosomally targeted, genetically encoded  $\text{Ca}^{2+}$ -sensor. *Biochem J* 2013; (449): 449-57.
40. Van Acker K, Bultynck G, Rossi D, Sorrentino V, Boens N, Missiaen L, et al. The 12 kDa FK506-binding protein, FKBP12, modulates the  $\text{Ca}^{2+}$ -flux properties of the type-3 ryanodine receptor. *J Cell Sci* 2004; (117): 1129-37.

41. Fameli N, Ogunbayo OA, van Breemen C, Evans AM. Cytoplasmic nanojunctions between lysosomes and sarcoplasmic reticulum are required for specific calcium signaling. *F1000Res* 2014; (3): 93.
42. Kinnear NP, Wyatt CN, Clark JH, Calcraft PJ, Fleischer S, Jeyakumar LH, et al. Lysosomes co-localize with ryanodine receptor subtype 3 to form a trigger zone for calcium signalling by NAADP in rat pulmonary arterial smooth muscle. *Cell Calcium* 2008; (44): 190-201.
43. Raffaello A, Mammucari C, Gherardi G, Rizzuto R. Calcium at the Center of Cell Signaling: Interplay between Endoplasmic Reticulum, Mitochondria, and Lysosomes. *Trends Biochem Sci* 2016; (41): 1035-49.
44. Morgan AJ. Ca<sup>2+</sup> dialogue between acidic vesicles and ER. *Biochem Soc Trans* 2016; (44): 546-53.
45. Bordi M, Berg MJ, Mohan PS, Peterhoff CM, Alldred MJ, Che S, et al. Autophagy flux in CA1 neurons of Alzheimer hippocampus: Increased induction overburdens failing lysosomes to propel neuritic dystrophy. *Autophagy* 2016; 10.1080/15548627.2016.12390031-17.
46. Kizilarslanoglu MC, Ulger Z. Role of autophagy in the pathogenesis of Alzheimer disease. *Turk J Med Sci* 2015; (45): 998-1003.
47. Chami M, Checler F. Ryanodine receptors: dual contribution to Alzheimer disease? *Channels (Austin)* 2014; (8): 168.
48. Zhang H, Sun S, Herreman A, De Strooper B, Bezprozvanny I. Role of presenilins in neuronal calcium homeostasis. *J Neurosci* 2010; (30): 8566-80.
49. Popugaeva E, Bezprozvanny I. Role of endoplasmic reticulum Ca<sup>2+</sup> signaling in the pathogenesis of Alzheimer disease. *Front Mol Neurosci* 2013; (6): 29.

## Figure legends

### **Figure 1: Inhibition of ectopically expressed RyR3 in HEK293 cells stimulates autophagic flux.**

(A) Left, immunoblot comparing HEK control cells to HEK RyR3 cells stained for the RyR, GAPDH and LC3. The used LC3-antibody recognizes both LC3-I (top band) and LC3-II (lower band). Right, quantification of the LC3-II levels obtained from different experiments. The averages  $\pm$  SD (n=5) are shown relative to the HEK control cells. Each data point represents an independently performed experiment. (B) Left, Immunoblot of HEK RyR3 cells treated for three hours with the vehicle (DMSO), 10  $\mu$ M dantrolene or 20  $\mu$ M ryanodine stained for the RyR, actin and LC3. When indicated bafilomycin A1 (100 nM) was added during the last hour of the treatment. Equal amounts of the vehicle (DMSO) were added to all samples. Right, quantification of LC3-II levels from the performed experiments in HEK RyR3 cells in the presence or absence of bafilomycin A1. Averages  $\pm$  SD (n $\geq$ 10) are shown relative to the vehicle control obtained in each experiment. Each data point represents an independently performed experiment. (C) Left, Z-projection of confocal image stack showing HEK RyR3 cells expressing mCherry-LC3 after the indicated treatments. Right, Quantification of mCherry-LC3 puncta, the average ( $\pm$  SEM) of five independently performed experiments is shown. Each data point represents the average of at least 10 cells.



**Figure 2: RyR3 inhibition does not alter the proximal/intermediate steps of autophagy**

Left, Immunoblots of lysates obtained from HEK RyR3 cells treated for three hours with the indicated RyR inhibitors or the vehicle control. Immunostaining was performed for proximal/intermediate autophagic markers. The antibodies used are indicated for each immunoblot. Right, Quantification of the performed experiments. Each data point represents an independently performed experiment. Averages  $\pm$  SD ( $n \geq 5$ ) are shown relative to the DMSO control treatment.

**Figure 3: Dantrolene and ryanodine inhibit spontaneous RyR3-mediated  $\text{Ca}^{2+}$  release in the conditions used for analyzing autophagic flux**

Single-cell  $\text{Ca}^{2+}$  measurements performed on OER-GCaMP6f-transfected HEK RyR3 cells.

A) Top, typical example of single-cell  $\text{Ca}^{2+}$  measurements performed after transfection with OER-GCaMP6f. Cells were treated for three hours with DMSO, dantrolene (10  $\mu\text{M}$ ) or ryanodine (20  $\mu\text{M}$ ) before starting the measurements. Spontaneous activity was measured in Krebs solution containing the indicated additions. In order to be classified as a bona fide spontaneous  $\text{Ca}^{2+}$ -release event, we established that the maximum peak amplitude of the  $\text{Ca}^{2+}$  release should be at least 0.02 units above baseline and/or should form a  $\text{Ca}^{2+}$  transient lasting longer than 5 secs. After measuring spontaneous activity for 5 min, RyRs were subsequently stimulated with 1.5 mM and 10 mM caffeine. Bottom, magnification of top panel highlighting showing spontaneous active cell during the first 5 minutes. B) Quantification of the performed experiments. The % of spontaneously active cells (grey part of the bar) was determined for each condition. Values indicate averages  $\pm$  SEM of three independently performed experiments (at least 150 cells were measured/condition).

**Figure 4: Dantrolene stimulates autophagic flux in differentiated C2C12 cells**

A) Left, immunoblot of undifferentiated (0 days) and differentiated (5 days) C2C12 cells treated for 3 hours with 10  $\mu$ M of dantrolene in the presence or absence of bafilomycin A1 (100 nM) during the last hour. Immunostainings were performed for LC3, RyR and GAPDH. Right, quantification of LC3-II levels in the performed experiments in the presence or absence of bafilomycin A1 (100 nM). Each data point represents an independently performed experiment. Averages  $\pm$  SD ( $n \geq 8$ ) are shown relative to LC3-II levels in the control treatment. B) Top, immunoblots for proximal/intermediate autophagic markers in C2C12 cells differentiated for 5 days treated for 3 hours with 10  $\mu$ M of dantrolene. Bottom, quantification of the performed experiments. Averages  $\pm$  SD ( $n \geq 3$ ) are shown relative to the control treatment. Each data point represents an independently performed experiment. C) Left, representative TEM images showing C2C12 cells differentiated for 5 days and treated for three hours as indicated. The white arrows indicate early autophagosomal vacuoles (scale bar: 500 nm). Right, quantification of the number of early autophagic vacuoles per cellular cross-section. Values indicate average  $\pm$  SD. The number of early autophagosomal vacuoles was counted in 10 randomly chosen cells per condition.

**Figure 5: RyR inhibition stimulates autophagic flux in dissociated hippocampal neurons**

Left, Immunoblot showing LC3 levels in lysates of 15 day-old dissociated hippocampal neurons treated for 3 hours with 10 or 20  $\mu$ M dantrolene. When indicated bafilomycin A1 (100 nM) was added during the last hour of the treatments. Right, Quantification of the LC3-II levels from the performed experiments in the presence or absence of bafilomycin A1. For each independently performed experiment (each data point), a new set of hippocampal neurons was isolated. Averages  $\pm$  SD (n=4) are shown relative to the control treatment.

Figure 1

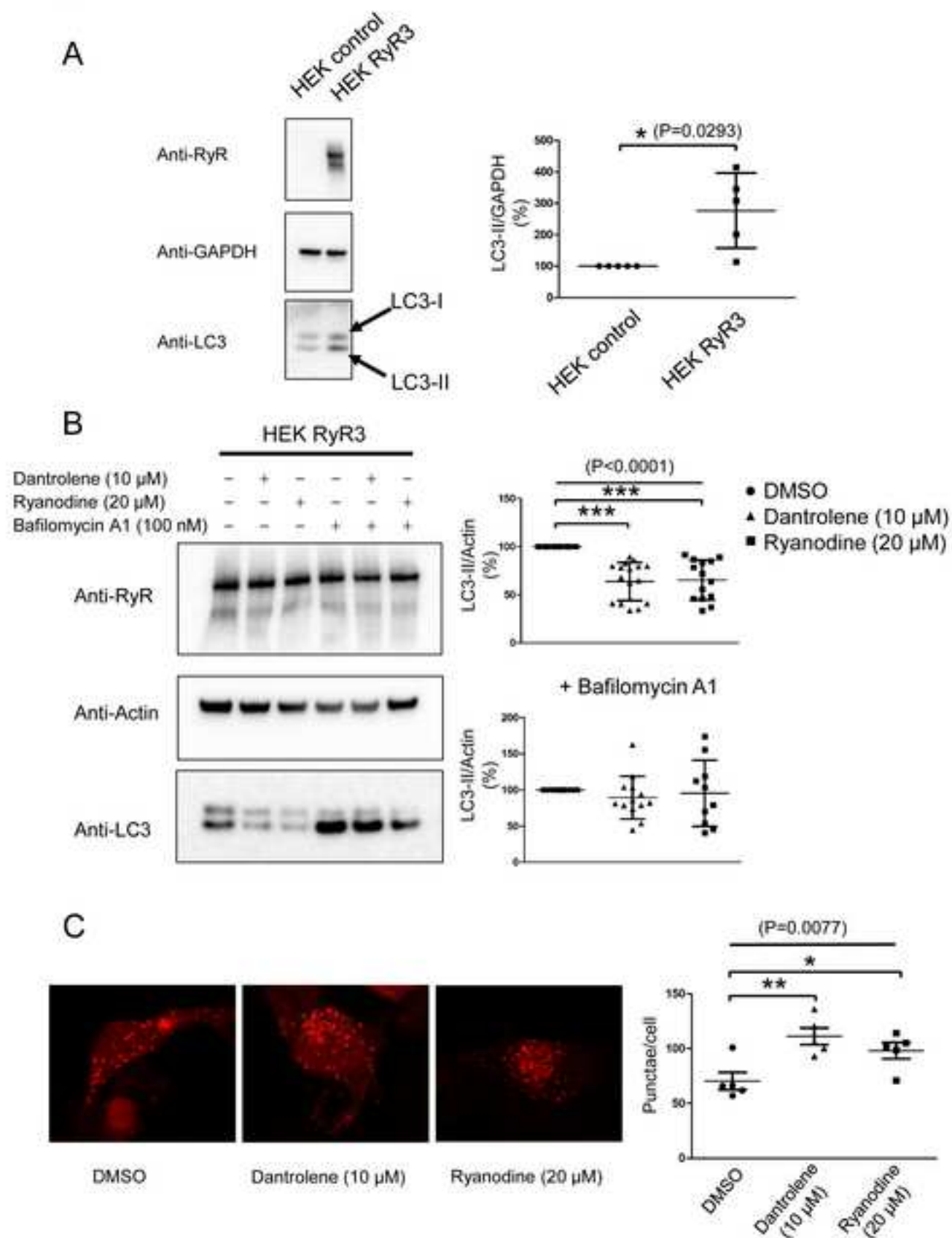


Figure 2

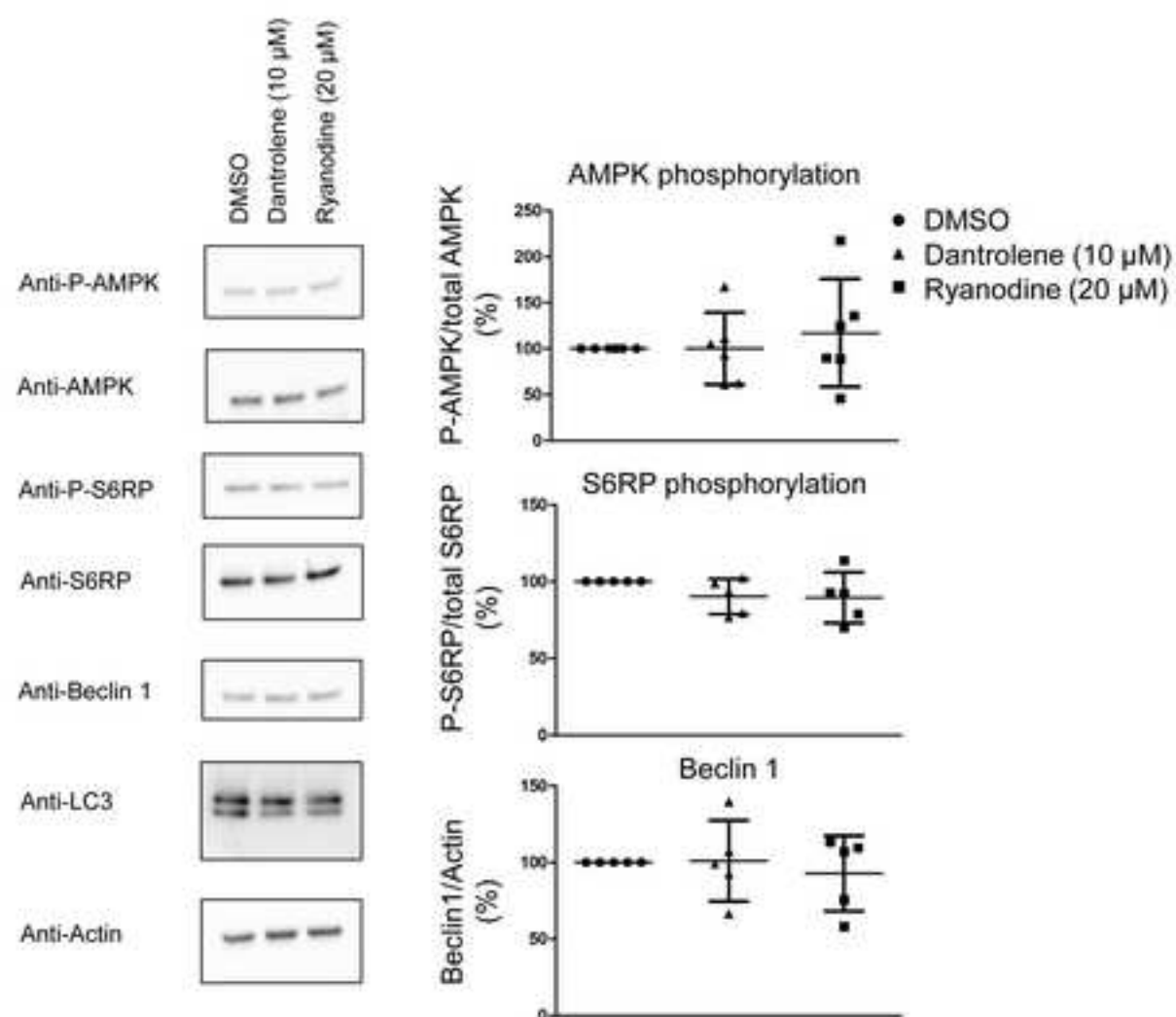


Figure 3

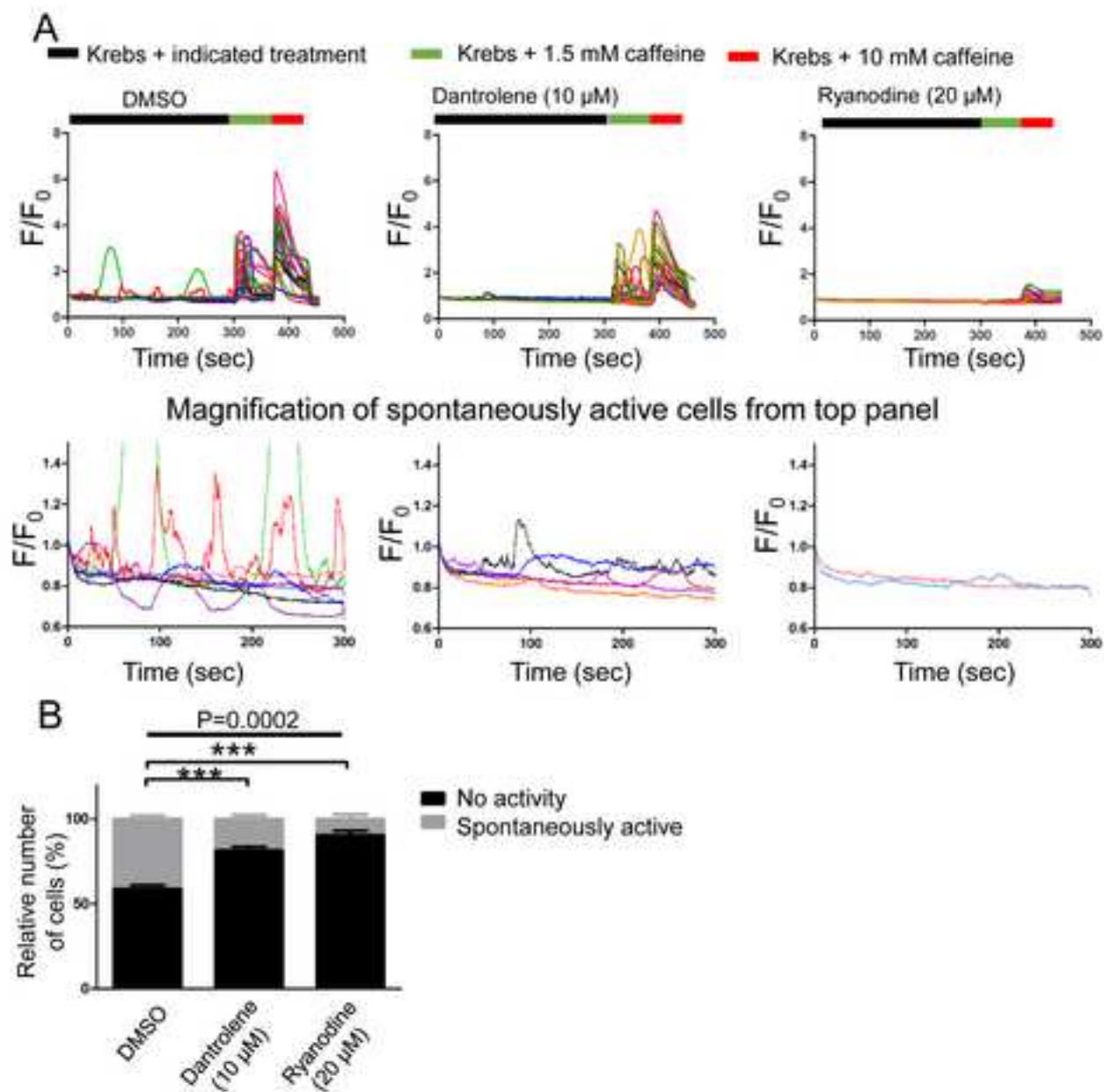


Figure 4

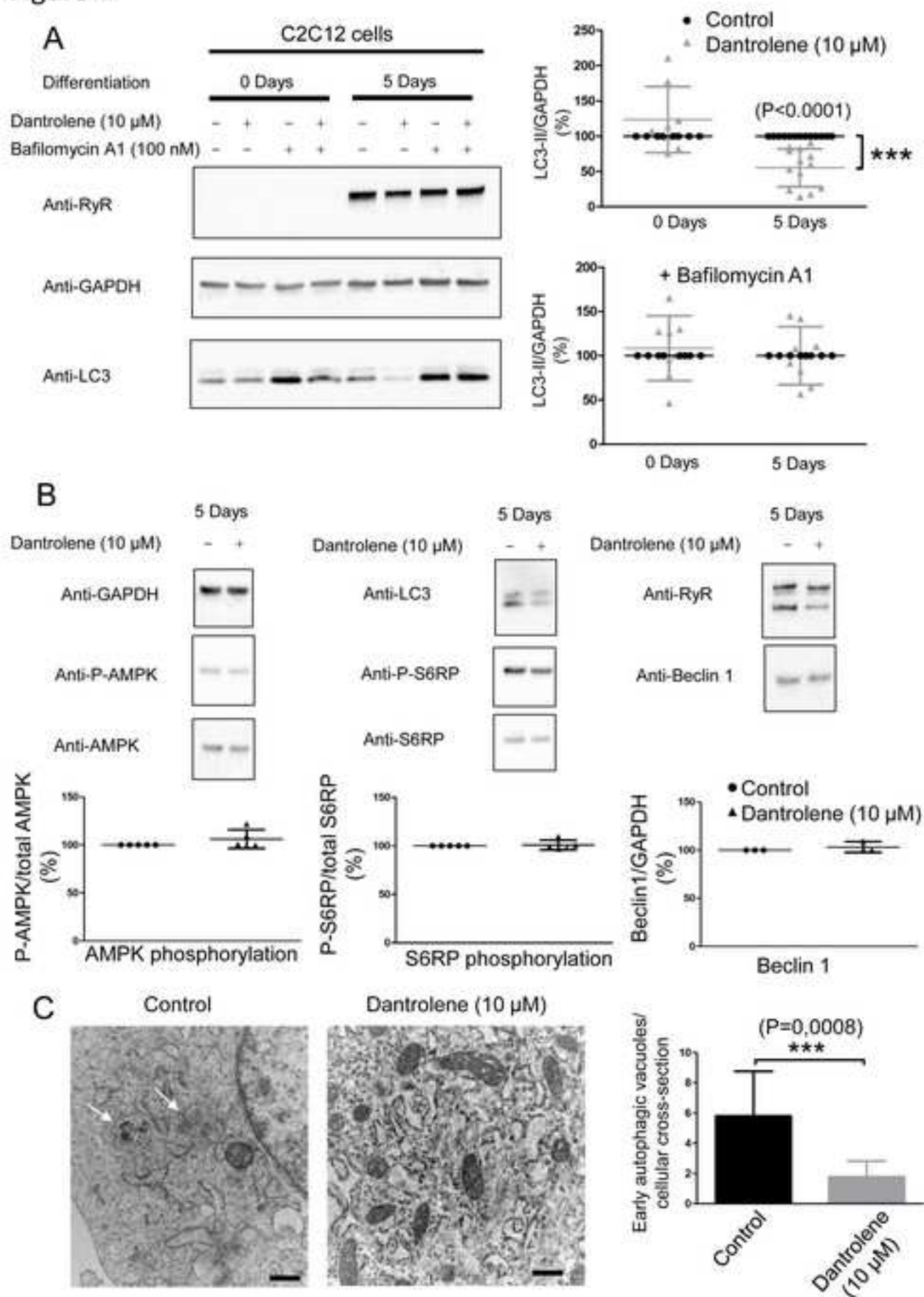




Figure 5

